An-Najah National University

Faculty of Graduate Studies

Pharmacological Effect and Chemical Compositions Variations of the Calamintha fenzlii Essential Oil Collected From Three Regions in Palestine

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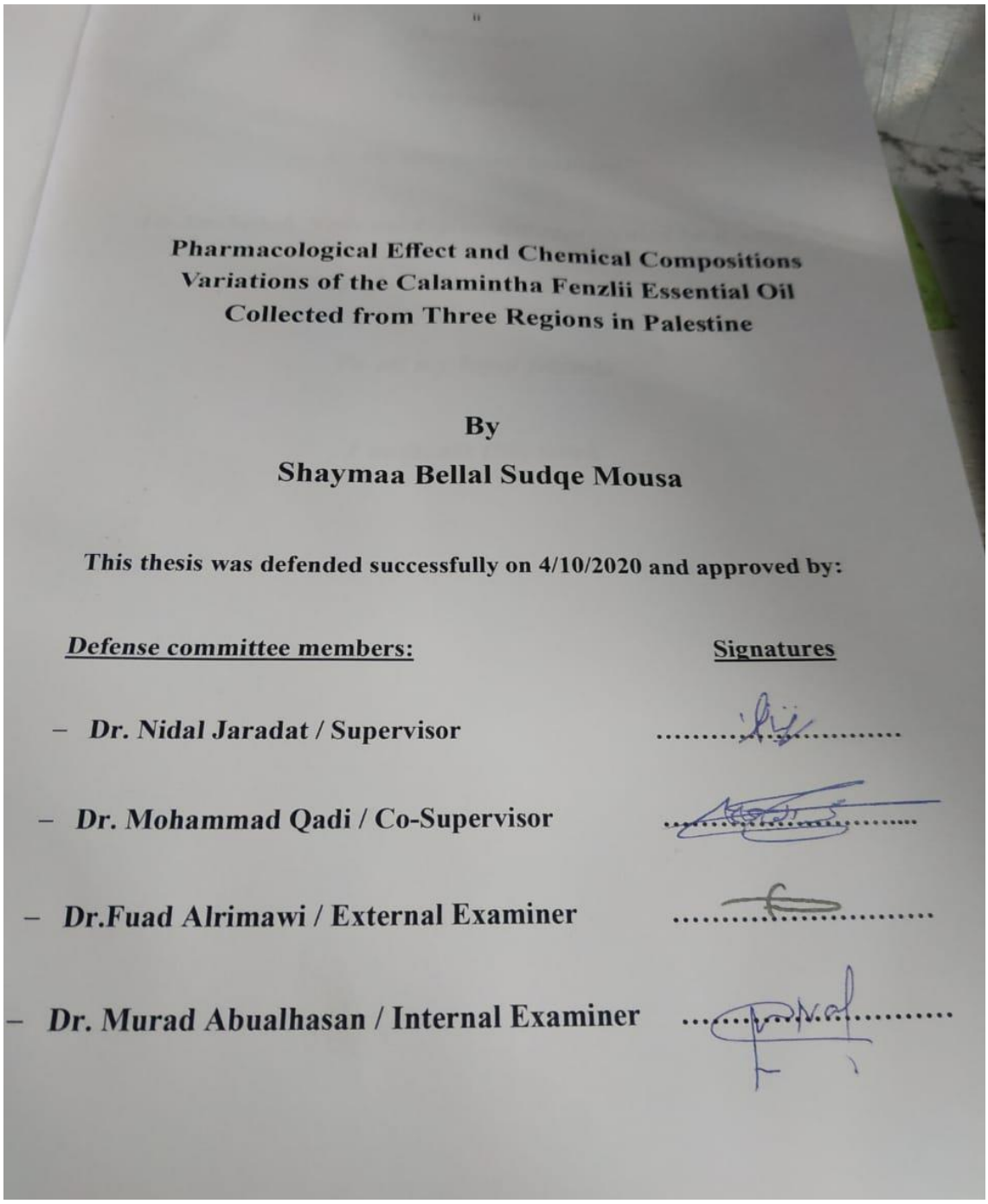

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Dedication

To my parents

To my sisters and brothers

To An-Najah National University represented by Rector;

Prof. Maher Al-Natsheh

To all my loyal friends

I dedicate this work

Acknowledgment

Thanks to my God who gives me the power to complete this work Thanks giving and estimate for help and prop are expanded to Dr. Nidal Jaradat and Dr. Mohammad Qadi for acceptance to be thesis supervisors, to be helpfulness, direction, discussions, advice, contribute to achieving this study.

I am thankful for the help and support of the laboratory team.

Deepest thankful to my family for support and help during my study.

الاقرار

انا الموقع ادناه مقدم الرسالة التي تحمل العنوان:

Pharmacological Effect and Chemical Compositions Variations of the Calamintha fenzlii Essential Oil Collected From Three Regions in Palestine

التأثير الدوائي واختلاف المكونات الكيميائية للزيت المستخلص من النعنع البري في ثلاث مناطق مختلفة في فلسطين

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اسم الطالبة:…… **Student's Name:……**

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List of abbreviation

Symbol	Abbreviation
ANNU	An-Najah National University
ATCC	American Type Culture Collection
BC	Before Christ
CAM	Complementary and Alternative Medicine
DMSO	Dimethyl Sulfoxide
GC-MS	Gas Chromatography/Mass Spectrometry
C. fenzlii	*Calamintha fenzlii*
CFW	*Calamintha fenzlii* Wadiqana
CFZ	*Calamintha fenzlii* Zawata
CFJ	*Calamintha fenzlii* Jnisinia
CFA	*Calamintha fenzlii* Auja
VRSA	Vancomycin-resistant *Staphylococcus aureus*
MIC	Minimum inhibitory concentration
MAP	Medicinal aromatic plants
DW	Distilled Water
MHB	Mueller Hinton Broth
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSA	Mannitol Salt Agar
NA	Nutrient Agar
NSAID	Nonsteroidal anti-inflammatory drug
EO	Essential Oil
VO	Volatile Oil
WB	West Bank
WHO	World Health Organization
NS	Normal saline
E. coli	*Escherichia coli*
K. pneumonia	*Klebsiella pneumonia*
P. vulgaris	*Proteus vulgaris*
P. aeruginosa	*Pseudomonas aeruginosa*
S. aureus	*Staphylococcus aureus*
C. albicans	*Candida albicans*
M. pulegium	*Mentha pulegium*
CFU	Colony-forming unit
R	Resistance

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Abstract

Background and Objectives

Calamintha fenzlii is a medicinal aromatic plant (MAP) with a pleasant minty fragrance. It is one of the most common MAPs in eastern Mediterranean regions, including Palestine. The objective of the current work was to screen and compare the chemical components and potential pharmacological properties of *C. fenzlii* essential oils (EOs) collected from three different regions of the West Bank, Palestine.

Methods

The EOs of four *C. fenzlii* samples were extracted by hydrodistillation. The EO samples were analyzed for chemical constituents using Gas chromatography-mass spectrometry (GC-MS). The antimicrobial activity was examined by the broth microdilution method. Eleven bacterial strains were tested, including seven Gram-positive strains (*Staphylococcus aureus* and six samples of methicillin-resistant *Staphylococcus aureus* [MRSA]) and four Gram-negative strains (*Proteus vulgaris*, *Pseudomonas*

aeruginosa, *Escherichia coli* and *Klebsiella pneumoniae*), in addition to one fungal strain (*Candida albicans*).

Results

GC-MS analysis revealed a high percentage of oxygenated components (range 97.54–99.18%) and a small percentage of non oxygenated components (range 0.74–1.64%), with the total identified compounds in each sample ranging from 98.28% to 99.9%. Several components were observed. Menthone was the most abundant component in four samples (range 14.83–93.83%) and pulegone was the most abundant in two samples (range 39.15–79.27%). Four EO samples exhibited broad antimicrobial activity, with three samples showing potent antifungal activity at minimum inhibitory concentrations (MICs) ranging from12.5 to 25 µl/ml, while one EO sample from Auja showed resistance to fungi. The sample from Wadiqana showed the highest potency against ATCC strians, with the lowest reported MIC value (12.5µl/ml), whereas the sample from Jnisinia demonstrated the lowest potency against MRSA, with the highest MIC value (50µl/ml). However, all four EO samples showed broad-spectrum antibacterial activity, with MIC values ranging from 12.5 to 50 µl/ml.

Conclusion

This study showed that *C. fenzlii* EO samples from different regions of Palestine contained different proportions of phytochemicals with

different biological activities, particularly antimicrobial activity, in line with the traditional use of *C. fenzlii* extracts. The plant extracts showed higher antibacterial potency compared to reference plants. Further *in vivo* studies are required to evaluate the potential pharmacological activity, safety and toxicity of this extract. Also, further studies are needed to isolate, identify and characterize the main components responsible for the potential pharmacological activity of *C. fenzlii* EO.

CHAPTER ONE

Chapter 1

1. Introductions

1.1 History of medicinal plants

Plants and their derivatives have long been used in cosmetics, as a preservative in food preparation and as a treatment in traditional medicine, and they are also used to manufacture the pure active ingredients of different drugs [1]. Herbal medicine (HM), also called botanical medicine, phytomedicine or phytotherapy [2] is economical, has minimal side effects and presents low environmental contamination, which makes it a good alternative therapy, especially in poorer communities. The Chinese were the first to use plants in traditional medicine, recorded since 4000–5000 BC. Later, as recorded in the Rigveda holy book, HM was used in India from 1600 to 3500 BC [3]. Archeological studies showed that herbal medicine existed as early as 60,000 years ago in Iraq and 8000 years ago in China [2]. More recently, the scientific community has returned to the investigation of medicinal herbs due to the lack of curative drugs for many chronic illnesses and to address the increasing resistance of modern drugs to many types of bacteria and other microbes. Modern medicines also have many side effects, in contrast to herbs [2].

The seeds, roots, leaves, fruit, bark, flowers, wood, berries and branches of plants, or even the entire plant, may be used in HM [2]. Several natural products that are important in modern medicine originated from

wild plants, wild microorganisms, novel organisms, and wild vertebrates and invertebrates [3]. The growing interest in natural products has led pharmaceutical companies to produce large amounts of herbal extracts and natural therapeutic agents [4].

In Indian medicine, also called Ayurvedic medicine, it is believed that everything can be medicine [2]. Nowadays, about 70% of the population of India use medicinal plants to treat disease, and approximately 25,000 products used in Indian traditional medicine consist of plants [2, 5].

In the Western world, including Europe and the USA, HM is used in complementary and alternative medicine (CAM) because these extracts are perceived to be natural and less harmful than synthetic drugs [5]. One study published in 2012 suggested that the use of CAM is strongly correlated with a higher level of education, and reported that its use has increased in younger patients with breast cancer [2]. Iraq was the first country in the Arab world to use herbs, with records dating back to 2600 BC. In Egypt, since 1500 BC, approximately 700 plants have been used as HM. Between 632 AD and 1258 BC, during the Arab Islamic Empire, more than 1400 herbal medicines were consumed by the Arabic population. In the Middle East, there are more than 2600 types of plants, but only about 250 of approximately 700 plants that are considered medicinal plants are used in Arab traditional medicine [2]. According to ethnopharmacologists, in the Mediterranean region, 250–290 herbal plants are still used today, especially in Palestine, where around 129 herbal plant types are consumed in Arabic

Traditional Medicine for the treatment of a variety of diseases, such as digestive diseases, liver diseases, cancer, respiratory diseases, skin diseases, and to lower “bad” cholesterol and combat a poor diet[2]. Medical plants are recognized for their potential use as drugs because they often have the same medical properties as synthetic drugs[6].

1.2 Current use of medicinal plants

Herbal medicine has been consistently used by a huge number of people from ancient centuries until now to meet the requirements of a healthy body and to help prevent illness. Around 80% of the Earth’s population use traditional HMs for their healthcare needs, such as plant extracts and their active ingredients, as reported by the World Health Organization (WHO) [1, 3]. Because HM is inexpensive and presents fewer side effects, many developed countries use traditional HM as an alternative to treat their health problems [7]. Drugs derived from plant extracts have an important role in the healthcare system of developed countries [1]. Phytochemical ingredients can support these medicines through a synergistic effect, and people can live for longer with a healthy body, which also increases their quality and quantity of life. In North America, there has been increasing attention on nutraceuticals and medicinal herb agents in the last year [8]. Furthermore, of all the drugs in the US market, 25% are HMs. Globally, the contribution of HM to the market is expected to increase from $ 61 billion USD to $ 5 trillion USD in 2025 [2]. In addition, pharmaceutical industries in China and Japan are interested in

making drugs derived from plant sources [1]. There are approximately 62 different therapeutic drug classes used worldwide, which came from 119 chemicals, extracted from 91 plant types [1]. Plant extracts and raw plants contain many phytochemicals and bioactive components with synergistic effects, which, unlike conventional drugs, can treat multiple illnesses [2]. The traditional use of medicinal plants is considered to be the major approach to the development of new drugs from natural sources [1]. The primary and secondary metabolites produced by plants are important for the growth and protection of the plant and are also of benefit to humans [9]. Primary metabolites like glucose, starch, polysaccharides, proteins, lipids, and nucleic acids are essential for the growth and development of the human body [6]. Secondary metabolites, including alkaloids, glycosides, flavonoids, phenols, steroids, saponins, tannins, terpenoids and essential oils are used for the treatment of diseases and are responsible for the therapeutic effects of plants. They have many therapeutic benefits for humans, for example, terpenoids and essential oils have anti-inflammatory, anticancer, anthelmintic, antimalarial, antiviral, antibacterial, cholesterol inhibition and insecticidal activities. Many drugs derived from bioactive compounds of medical plants are used to treat disease: digoxin from *Digitalis lanata*, which is used to treat arrhythmia; quinine from *Cinchona robusta*, for its antimalarial and antiparasitic effects; artemisinin from *Artemisia absinthium* for its antimalarial properties; tannic acid and tannins from *Terminalia arjuna* for their cardioprotective, anticancer and

hepatoprotective activities; mono- and sesquiterpenoids from *Zingiber officinalis* (ginger) for their anticancer, antioxidant, hepatoprotective, hypercholesterolemic and antiatherosclerotic effects; aloin and emodin from *Aloe vera* for their healing properties, antiviral, antitumor, antidiabetic, hepatoprotective and antiseptic effects; piperidine from *Piper nigrum* for its anticancer, antihyperlipidemic and antiepilepsy effects; tannins and shikimic acid compounds from *Terminalia chebula* for their antioxidant, antidiabetic, retina-protective and hepatoprotective effects; alkaloids (etoposide and teniposide) from *Podophyllum peltatum* for their anticancer effects; alkaloids (vinblastine and vincristine) from *Catharanthus roseus*as anticancer agents; topotecan and irinotecan from *Camptotheca acuminate* as anticancer agents (treatment of ovarian and small cell lung cancers); and limonoids (nimbidinin), di- and triterpenoids from *Azadirachta indica* for the prevention of carcinoma and colon cancers, and as chemopreventive, antiallergic and blood purifier agents [6, 10]. For centuries, people have returned to natural products to treat colds, allergies, upset stomachs, and toothaches. There has been a global trend toward a shift from synthetic to natural HMs for the prevention and treatment of diseases, a so-called ‘return to nature’ [6]. Some plants may be harmful to humans because they contain toxic compounds [6]. Nearly 122 compounds extracted from medicinal plants are used in modern medicine, and for 80% of these components, their active ingredient is currently used in a similar way to its traditional medicinal use [2]. When cultivated under

suitable geographical and environmental conditions, harvested in the appropriate season and collected from the right parts of the plant, medicinal plants contain a large amount of their active ingredient, which improves the economic worth of the plant [1]. The broad use of natural medical plants in new medicine indicates the applicability of their components to the treatment of many diseases [2]. The variety of chemical structures of compounds used in semi and total synthesis of new chemical products mean that they can be used many times for the treatment of many diseases without concern about side effects [3].

1.3 Essential oils

Essential oils (EOs) are also called ethereal oils or volatile oils because they can easily oxidize due to heat, light, and air, and have different actions depending on their chemical composition [11]. Essential oils are aromatic and volatile liquids extracted from various sections of plants (seeds, roots, bark, buds, leaves, wood, flowers, fruits, twigs, blossoms and herbs) using different processes. The method of extraction depends on the volatility of oil and plant origin. For example, orange and lemon oils are extracted by simple pressing; mustard and bitter almond oils are extracted by fermentation followed by distillation extraction, and the most commercially common used method is steam distillation [12]. In the past, in Egypt, oils were extracted by infusion. Many years later, the Greeks and Romans extracted oil by distillation to give odorous plants extra worth. In the age of Islamic civilization, extraction techniques have

been further refined [11]. EOs are soluble in alcohol, hydrophobic non-polar or weakly polar solvents, waxes, and oils. Compounds of EOs can cross the blood-brain barrier due to their lipophilic characteristic and small size. EOs are usually a pale yellow liquid with a lower density than water, or they may even be colorless; however, chamomile (*Matricaria chamomilla*) is the one exception because its EO is blue [11]. EOs have been traditionally used and are still used in foods and beverages as spices or herbs, as preservatives, in cosmetics such as perfumes, aftershaves and fragrances, in addition to their agricultural applications. EOs are also used to treat the body and mind as alternative medicine [11, 13, 14]. EOs are used as medicine because of their antioxidant activity, which neutralizes reactive oxygen species that increase under specific pathological conditions, leading to oxidative stress, which is considered a major risk factor for cancer, cardiovascular disease, type2 diabetes and neurological diseases [4]. EOs are natural antioxidants, so they are good alternative to synthetic antioxidants, which have carcinogenic effects [4]. EOs have anti-inflammatory activity through different mechanisms of action, including the production of prostaglandins [4]. Because they act as a selective COX-2 inhibiter, EOs can be a good alternative to Nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit both COX-1 and COX-2 and cause undesirable gastrointestinal side effects [4]. There are almost 3000 different EOs, but only about 300 are used commercially in the flavoring and fragrance markets [15]. Four properties are important to know when using

EOs as food preservatives: (1) the minimum inhibitory concentration (MIC), (2) the range of target organisms, (3) the mode of action, and (4) the effect of food matrix components on their antimicrobial properties [15]. There are two main barriers to use of EO components as food preservatives: (1) they are not potent enough as single components; and (2) when added in sufficient amounts to provide an antimicrobial effect, they cause negative organoleptic effects [15].

There are three ways for an EO to enter the body: direct absorption through inhalation, diffusion through the skin tissue, or ingestion. Some EOs have a physiological effect, for example, fennel contains an estrogen-like compound that is effective for female problems such as pain of menstruation and lactation, and other EOs have a psychological effect, like lavender and lemon, as they have sedative and relaxant properties. Nowadays, peppermint, chamomile, lavender, tea tree, eucalyptus, jasmine, rose, lemon, orange, rosemary, bergamot, geranium and sandalwood EOs are the most frequently used [11]. Odorous and volatile compounds are found in only 10% of all plants worldwide, and these compounds are present and stored in plants in secretory structures, such as glands, secretory hairs, secretory cavities, secretory ducts, or resin ducts. Most plants have a very low total EO content (1%), but in some cases, for example, clove (*Syzygium aromaticum*) and nutmeg (*Myristica fragrans*), this percentage reaches more than 10% [11]. There has been a rapid increase in EO production and consumption around the world despite their

elevated cost (due to the huge amount of plant material required). The worldwide production of EOs ranges from 40,000 to 60,000 tons per year, with a market of approximately $ 700 million USD [11].

There are two classes of bacteria: Gram-negative bacteria and Gram-positive bacteria. Gram-negative bacteria contain hydrophilic lipopolysaccharides (LPS) which work as a barrier to prevent macromolecules and hydrophobic compounds. As a result, Gram-negative bacteria are more tolerant of oils containing hydrophobic antimicrobial compounds [15]. EOs are categorized into phenylpropanoids and terpenoids or hydrocarbons (limonene, pinene, myrcene, sabinene, cymene and phellandrene) and oxygenated compounds (ketone, menthone, pulegone, fenchone and camphor), alcohols (linalol, menthol, borneol and santalol), phenols (thymol, eugenol and carvacrol), among others [11, 13]. These compounds can be classified into four groups depending on their chemical structure: terpenes, terpenoids, phenylpropenes, and “others” [15].

Terpenes are hydrocarbons produced from the conjunction of different isoprene units (C_5H_8) (Figure 1). The main terpenes are monoterpenes ($C_{10}H_{16}$) and sesquiterpenes ($C_{15}H_{24}$), and longer chain terpenes include diterpenes ($C_{20}H_{32}$) and triterpenes ($C_{30}H_{48}$). Among the examples of terpenes (limonene, pinene and p-cymene), p-cymene, one of the major components of thyme, was found to have no antimicrobial

activity against many Gram-negative bacteria, suggesting that terpenes are not active as antimicrobials when given as single compounds [15].

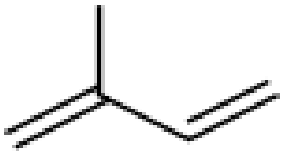

Figure 1: Isoprene (C_5H_8) [16].

Terpenoids, also known as isoprenoids, are terpenes produced by the addition of oxygen molecules or by moving or removing methyl groups by enzymatic biochemical modifications. They consist of a number of isoprene units (C_5) linked from head to tail (Figure 1) [15]. Terpenoids can be subdivided into aldehydes, alcohols, ethers, epoxides, phenols and ketones [15]. Terpenoids are antimicrobial compounds that act against a broad spectrum of microorganisms, with carvacrol and thymol found to be the most active monoterpenoids against bacteria [15].

Phenylpropanoids are found in small amounts in EOs and are derived from shikimic acid (Figure 2) [16]. They contain a bioactive constitute created by plants to protect themselves from herbivores, wounds, infections and ultraviolet irradiation [17]. Examples of phenylpropanoids include eugenol, vanillin, safrole and cinnamaldehyde [15].

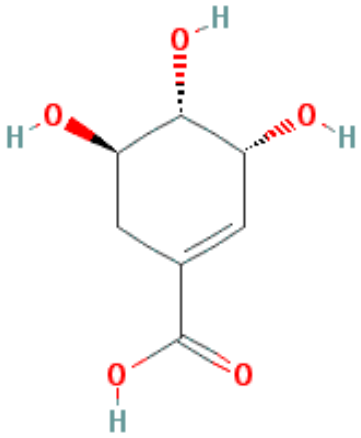

Figure 2: Shikimic acid ($C_7H_{10}O_5$) [18].

Other EO constituents include products of the degradation of original compounds like terpenes, glycosides, lactones, unsaturated fatty acids and sulfur-and nitrogen-containing compounds (allyl isothiocyanate [AITC] and allicin) [15].

The odor and chemical composition of EOs may vary depending on the growth conditions (amount of water, climate, type of soil and soil composition, altitude), plant age, environmental conditions, collection time, and the geo-climatic location and site of growth [19].

The therapeutic activities of EOs are determined by their chemical structure, and include antiviral, antimicrobial, antiseptic, anesthetic, cell-regenerating, digestive, vasodilator, hypotensive, calming, sedative, spasmolytic, antipyretic, expectorant, stimulant, tonic, antitumor, analgesic and anti-inflammatory effects [11].

The genetic composition is an important factor that affects the chemical composition of the EO. This may lead to the production of a similar EO that differs in its chemical composition, even though it is from the same part of the plant. Different therapeutic activities may result from these differences in chemical composition. Variations in chemical composition are known as chemotypes. For example, peppermint (*Mentha piperita* L.) has three chemotypes: menthol, carvone, and limonene[11].

1.4 Background

1.4.1 *Calamintha*

The genus *Calamintha* belongs to the *Lamiaceae* family. Commonly called calamint, there are many species of *Calamintha*: *Calamintha ashei, Calamintha baumgarteni, Calamintha dentata, Calamintha grandiflora* (large flowered calamint, an ornamental plant), *Calamintha caerulescens, Calamintha coccinea, Calamintha incana, Calamintha nepeta (Calamintha nepeta* subsp. *Nepeta*), *Calamintha fenzlii,* and *Calamintha sylvatica* (common calamint, a low growing plant with a minty smell and lavender flowers). *Calamintha* species are used as a food source for larvae of some Lepidoptera, especially *Coleophora albitarsella*

1.4.2 *Calamintha fenzlii*

1.4.2.1 Scientific name, synonyms and traditional names

As a medicinal aromatic plant (MAP) from the *Lamiaceae* family, *Calamintha fenzlii* is commonly known as pudding grass, mosquito plant and squaw mint [4]. Its scientific name is *Calamintha fenzlii*, and its synonyms are *Mentha pulegium* L, *Melissa pulegium* (L) Griseb, *Mentha gibraltarica* Willd, *Satureja fenzlii* and *Mentha aromatic* Salisb. In Palestine, it is commonly known as wild mint.

1.4.2.2 Description of *Calamintha fenzlii*

Calamintha fenzlii is a grassy and flowering perennial plant that is local to eastern parts of the Middle East, central and southern Europe, eastern regions of Asia, and northern regions of Africa. It is15 cm in height, with an unknown width because of its fast-growing and spreading nature. It has dark green colored oval leaves with small hairs on both sides and toothed margins; the flower color is mauve (Figures 3 and 4), and flowers are tiny, appear late in spring and grow up the square stem and spread out from near the node of the leaf [4].

Figure 3: *Calamintha fenzlii* plant

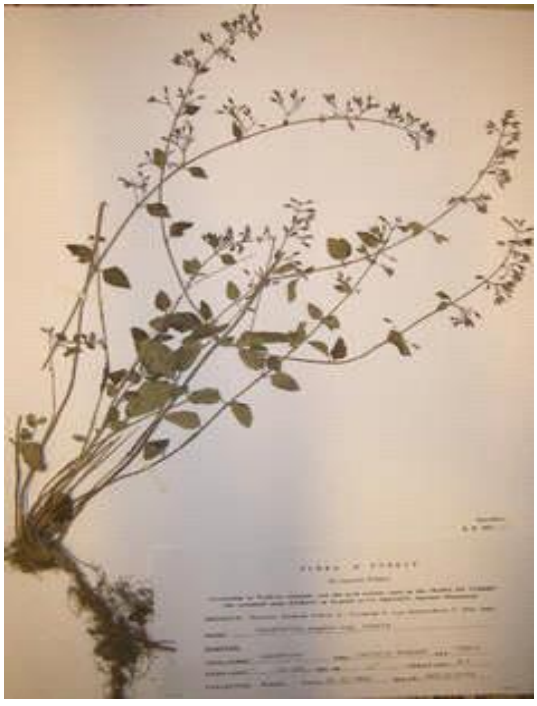

Figure 4: *Calamintha fenzlii* plant

1.4.2.3 Folk uses

In traditional medicine, *C. fenzlii* is used for indigestion, cough, fever, kidney and liver problems and headaches, and for the treatment of tuberculosis (TB), influenza and smallpox [4].The fresh or dried leaves and flowering tops are commonly used for their healing and culinary properties. The whole plant and its volatile oil have a strong smell.

Early settlers in colonial Virginia

Pennyroyal was popularly used by early settlers in colonial Virginia against rattle snake bites and for its antibacterial and antioxidant effects. It has also been traditionally used as an emmenagogue, abortifacient, and to relieve flatulence and an upset stomach. The leaves, fresh or dried, were traditionally used for abdominal cramps, to induce sweating and promote latent menstruation, as well as for the treatment of influenza, colds, smallpox and TB. However, excessive use must be avoided due to the toxicity of a pregnant woman in Colorado who died one week after taking 1 ounce of concentrated pennyroyal oil to self-induce an abortion. It has antihepatotoxicity, antibacterial, acaricidal, antioxidant, antimicrobial, relaxant and spasmolytic effects [20].

Palestine

In Palestine, wild mint leaves are boiled in water, and 2–4 cups a day are consumed for its sedative and antispasmodic effects and for treating muscle spasms and convulsions [21].

Jordan

In Jordan, it is used to treat arthritis and hyperglycemia, and the mentha branches are used to relieve flatulence and neutralize acidity [22].

Turkey

The*C. fenzlii* plant is used as a herbal tea in Turkey for its antibacterial, antifungal, antipyretic, antihistaminic, antitreponemal, anticonvulsant, hepatoprotective, antihypercholesterolemic, cytochrome P-450 inhibition and lysosomal enzyme inhibition activities. It is not mutagenic, but it can be lethal as pulegone can cause hepatotoxicity. However, there have been no reports of death caused by the ingestion of oil containing pulegone. There was one reported case of a pregnant woman who lost consciousness half an hour after consuming *C. fenzlii*, followed by delirium for 4 hours, extensive necrosis of the liver and shock due to cardiorespiratory arrest; however, after vomiting, complete recovery was observed [23].

Morocco

In Morocco, it is traditionally used for its antispasmodic, carminative, diaphoretic, diuretic, sedative, antitussive, stimulant, tonic, expectorant, antiseptic and cholagogue effects, and also to promote menstruation, cure headaches, treat bronchitis, relieve bites from snakes and scorpions, and to treat acne and other skin conditions. It is also used to relieve vomiting and for kidney disease. It also serves as an evictor of fleas and insects. *C. fenzlii* is used in cosmetics and as a spice and flavoring in different foods, especially candy, as well as an abrasion inhibitor for steel in the chemical industry. A small amount can cause hepatotoxicity, which can lead to severe poisoning, and a large amount can cause fatal liver necrosis. Therefore, the correct dosage and administration of the plant are important to avoid accidents. Thymol and carvacrol are considered effective natural antimicrobial agents [24].

1.4.2.4 Chemical composition

The chemical composition and percentage of specific components in the EO of *C. fenzlii* may vary depending on differences in cultivation, origin, growing season and the vegetative stage of the plants. The major component of the EO and aqueous extract of the plant was found to be monoterpenes (pulegone, menthol, isomenthol, isomenthone, limonene, α-pinene and β-pinene) [25].

1.4.2.5 Evidence-based use

The oil extract of *C. fenzlii* showed clear anticancer activity against two human breast adenocarcinoma (MCF-7 and T-47D) and one human colon cancer (Caco-2) cancer cells lines [26]. It showed the strongest inhibition power in the butyrylcholinesterase (BuChE) inhibition assay (anti-tyrosinase activity) [26]. Its use ranges from food preservation, due to its antioxidant activity, to possibly aiding in therapy for Alzheimer's disease and in cancer treatment [26]. Oil extracts can be used to protect against gastric ulcers because they have an anti-inflammatory effect, so they can be used as a safe alternative to NSAIDs, which cause gastric ulcers because they have no side effects [4]. The EO of *C. fenzlii* also shows antibacterial, antifungal, antipyretic, antihistaminic, antitreponemal, anticonvulsant, hepatoprotective, antihypercholesterolemic, cytochrome P-450 inhibition and lysosomal enzyme inhibition activities. It is not mutagenic, although pulegone may cause neuro- and hepatic toxicities, which may be lethal [23]. It has antioxidant activity, so it may be used as palliative therapy for liver injury and has a clear effect on the inhibition of inflammatory pain [4]. It can be used as a natural medium to replace synthetic herbicides due to the presence of pulegone at a high concentration [25].

1.5 Problem statement

Resistance to antimicrobial agents occurs when microorganisms like bacteria, viruses, fungi and parasites adapt and develop resistance to these agents due to exposure and frequent misuse of antimicrobial medicines like antibiotics, antivirals, antifungals and antiparasitics. Therefore, these drugs become less effective and infections become more prevalent [27]. Microorganisms that have resistance properties are called “superbugs” [27]. At least 2 million people in the USA are infected with bacteria that are resistant to antibiotics, and more than 23,000 people die per year as a result [27]. With time, microorganisms naturally develop antimicrobial resistance far away from the variable in genetic changes [27]. The overuse and incorrect use of antibiotics in humans, animal farms and in the environment, such as the use of antibiotics to treat viral infection (flu or cold) or to stimulate animal growth without formal prescription, leads to the rapid development of antimicrobial resistance. Inappropriate health conditions, food handling and infection control are all factors that increase the prevalence of antimicrobial resistance [27]. Worldwide, microorganisms are constantly developing resistance mechanisms and, as a result, the prevalence of antimicrobial resistance has increased [27]. Prolonged disease, treatment failure, and death resulting from antimicrobial resistance are threats to human life [27]. Because of this, antimicrobial resistance is considered a global concern [27]. For cancer chemotherapy, diabetes management, organ transplantation and surgeries like hip

replacement and cesarean, the absence of antimicrobial medicines for protection and treatment may become a serious hazard [27]. In addition, expenses related to hospitalization and health care greatly increase with antimicrobial resistance, as many patients require intense care [27]. Antibiotic-resistant infections require prolonged hospital stays, more intensive follow-up through doctor visits, and expensive [27]. Because of the spread of antimicrobial resistance and the associated expense, continuous development will be highly needed [27]. The complications associated with multidrug resistance have increased the pressure on experts to look for new antimicrobial agents from diverse sources, such as medicinal herbal plants [28]. Every year in the US, a minimum of 2.8 million people will have an antibiotic-resistant infection, and more than 35,000 people will die. In addition, people with chronic illnesses have a greater risk than others [27]. For thousands of years, aromatic plants have been used as preservatives due to their antioxidant effect, and they may also have medicinal uses. Nowadays, there is growing interest in the extraction of EOs from medicinal plants for the development of alternative therapies and to prevent or delay the growth of pathogens [29]. In developing countries, 7% of people have drug-resistant HIV upon starting antiretroviral therapy, also present in more than 15% of people who have already began HIV treatment and nearly 40% of people who are restarting treatment [27]. All influenza A viruses are resistant to one class of antiviral drugs—M2 inhibitors (amantadine and rimantadine) [27]. *Mycobacterium*

tuberculosis that are resistant to the two most powerful anti-TB drugs are called multidrug-resistant tuberculosis (MDR-TB), while TB strains that are resistant to at least four of the core anti-TB drugs are called extensively drug-resistant tuberculosis (XDR-TB) [27]. The resistance of *Klebsiella pneumonia* to carbapenems has spread around the world. These intestinal bacteria are the main cause of hospital-acquired infections, such as bloodstream infections, pneumonia and other infections in newborns and intensive care unit patients [27]. The resistance of *E. coli* to fluoroquinolone, which is used to treat urinary tract infections, has also spread around the world. The resistance of gonorrhea to third-generation cephalosporins has been reported in 10 countries. Methicillin-resistant *Staphylococcus aureus* (MRSA), which became resistant in 1960, is widespread and has a 64% higher mortality rate than non-MRSA *S. aureus* [27]. Colistin is the drug of choice when carbapenem-resistant *Enterobacteriaceae* emerges. Colistin-resistant bacteria cause many untreated infections, and are spread across many countries [27]. Penicillin was the first antibiotic, discovered in 1928 by Alexander Fleming. Later, in1942, *S. aureus* developed resistance to penicillin. Penicillin-resistant *Streptococcus pneumonia* appeared in 1967 [27]. This was followed by the first vancomycin-resistant *Staphylococcus aureus* (VRSA) in 2002, amphotericin B-resistant *Candida auris* in 2016, azithromycin-resistant *Neisseria gonorrhoeae* in 2011, ciprofloxacin-resistant *N. gonorrhoeae* in 2007, caspofungin-resistant *Candida* in 2004, daptomycin-

resistant MRSA in 2004 and ceftazidime-avibactam-resistant KPC-producing *K. pneumonia*in 2015 [27].

As mentioned in the above problem statement, antimicrobial resistance leads to the development of serious diseases in humans and results in increased levels of mortality around the world. The universal direction toward natural products is increasing since herbal plants have a wide range of chemical compounds that may have a synergistic effect in treating diseases with lower toxicity and fewer side effects. The variety of chemical compounds found in the EOs of aromatic herbal plants is related to different variables, such as the part of the plant used for extraction, the soil, the season and the climate. The study of traditional herbs used to treat different diseases in the past is encouraging. Thus, the purpose of this study was to define the chemical composition of the EOs of *C. fenzlii* and their characteristics.

1.6 Objectives of the study

1.6.1 General objective

The main aim of this thesis was to compare the chemical composition of *C. fenzlii* EOs collected from three geographical regions in Palestine, namely Zawata and Jnisinia (north), Wadiqana (center) and Auja (south), and to screen the potential biological activities and pharmacological properties of these EOs.

1.6.2 Specific objectives

In the current study, *in vitro* screening of the potential antimicrobial activities (antibacterial and antifungal) of the EOs was performed and compared. There were three specific objectives of this thesis:

- To analyze the chemical composition of *C. fenzlii* EOs using GM-MS.
- To investigate the antibacterial and antifungal activities of *C. fenzlii* EOs.
- To conduct a comparative study of the findings of the aforementioned tests for EOs of *C. fenzlii* from three geographical regions in Palestine.

1.7 Significance of the study

This is the first study to assess the chemical composition and biological activity of EOs of *C. fenzlii* collected from three regions in Palestine with different geographical locations. Consequently, the study may be a valuable tool in the following ways:

- Explore the chemical constituents of *C. fenzlii* EOs.
- Determine whether there are differences in the chemical constituents of *C. fenzlii* EOs from different regions of Palestine.
- Investigate the biological activities (antibacterial and antifungal) of *C. fenzlii* EOs from different regions of Palestine.

- Serve as a tool to select the most suitable oil for use as traditional medicine for treating disease more effectively and efficiently depending on the chemical composition and concentration of important constituents of *C. fenzlii* EOs from different regions in Palestine.
- Provide information about the most suitable environmental and geographical conditions for commercial agricultural cultivation.
- Add economical value to *C. fenzlii* EOs produced in Palestine.

CHAPTER TOW

CHAPTER 2

2. Materials and methods

2.1 Materials

The materials used in the study were of analytical grade and used without further purification. Dimethylsulphoxide (DMSO) 100% was purchased from CARLO ERBA (France) for use in the antimicrobial screening assay.

2.1.1 Materials used in the production of essential oils

The calcium carbonate used to dry the EOs was purchased from Sigma-Aldrich (USA).

2.1.2 Materials used for antimicrobial screening

2.1.2.1 Antibacterial screening

Mueller Hinton Broth (21.9 g/L) and nutrient agar (28.0 g/L) were purchased from (Hi Media Laboratories, Mumbai, India).

2.1.2.2 Antifungal screening

Ready-to-use sterile RPMI 1640 medium with L-glutamine was used to culture *Candida albicans* in this study. The medium was purchased from Biological Industries (northern Kibbutz Beit Haemek, Israel).

2.2 Instruments

2.2.1 Essential oil extraction and chemical screening

Hydrodistillation was used for the extraction of EOs. Balance max 220 g (Radway, Poland) was used to weigh the plant material. Gas chromatography-mass spectrometry (GC-MS; QP-5000; Shimadzu, Japan) was used for the chemical screening of EOs.

2.2.2 Antimicrobial screening

Balance max 300 g (d=0.001g,AY 303) was purchased from Sartorius (Canada). Other equipment included a heater (Lab-Tech, Korea), autoclave for sterilizing media, water and disposed materials (MRC, Palestine), Bunsen burner (Ningbo I.G.I Gas Industry, China) and hood (BIOBASE, China) for working under aseptic condition, in addition to the refrigerator (Ariston, USA), water bath and incubator (Ari j Levy, Haifa). A 30–300-µl multichannel micropipette (MRC, Haifa), 100–1000-µl single micropipette (Microliter, BRAND, Germany), 20–200-µl single micropipette (Huma pette, Germany) and white, yellow and blue pipette tips were used to measure the minute volumes of plant extract. For cell culture, 96-well microplates were purchased (Greiner bio-one CELLSTAR, Austria). Disposable sterile syringes (5 and 10 ml; Changzhou Heany, Jiangsu, China) and 0.25-µm sterile syringe filters (KDL, China) were purchased, in addition to other equipment like large and small glass test tubes, large and small plates, loops, disposable sterile pipettes (1, 5 and 10

ml), Eppendorf tubes (Nichipet EX, Japan), autoclave sterilization tape and parafilm M (Bemis, USA). A UV-visible spectrophotometer (Jenway 7315, England) was used to measure the turbidity of bacterial and fungal cultures diluted with normal saline.

2.3 Methods

2.3.1 Plant material collection and preparation

The aerial parts of *C. fenzlii* were collected in 2020 from four locations in the West Bank (WB) of Palestine—Zawata, Jnisinia, Wadiqana, and Auja—representing the north, middle and south regions of the WB of Palestine, respectively. The samples were botanically identified and coded by Dr. Nidal Jaradat, the pharmacognosist at An-Najah National University (ANNU). The extraction of EOs was performed following a previously described method [30]. The fresh aerial parts of *C. fenzlii* were carefully separated, washed two times with distilled water, dried for two weeks in the shade at room temperature, and stored in sealed plastic bags for future use in the Laboratory of Pharmacognosy at the Faculty of Medicine and Health Sciences.

2.3.2 Essential oil extraction

Isolation and characterization of *Calamintha fenzlii* essential oil

The EOs of *C. fenzlii* plants collected from three regions of Palestine were separated by hydrodistillation [31]. Briefly, 0.1 kg of the dried leaf

powder was suspended in1 L of distilled water, then the EO was extracted using hydrodistillation with the Clevenger apparatus operating at atmospheric pressure for 90 min at 100°C. This process was repeated four times for each plant sample. The obtained EOs were chemically dried using calcium carbonate and stored at 2°C until further use.

2.3.3 Gas chromatography-mass spectrometry

The EOs were characterized with the GC-MS method, performed using a Perkin Elmer Clarus 500 GC gas chromatograph equipped with a Perkin Elmer Clarus 560 mass spectrometer. The separation was achieved using a Perkin Elmer Elite-5 fused-silica capillary column (30 m × 0.25 mm, film thickness 0.25 µm). The column temperature was programmed to increase from 50°C for 5 min to 280°C at a rate of 4°C/min. The flow rate of helium as the carrier gas was 1 ml/min, which was kept constant for the entire chromatographic run. Neat oil (0.2 µl) was injected in split mode with a split ratio of 1:50 and at a temperature of 250°C. The sample components were identified by matching their mass spectra with those of the library or to spectra of pure standard components confirmed by their GC retention times [32].

2.3.4Antimicrobial screening

2.3.4.1 Microorganisms and conditions for cultivation

Bacterial strains

The *C. fenzlii* EO samples were studied for their antimicrobial activities. The antibacterial activities of EOs were investigated against the growth of five reference bacterial strains obtained from the American Type Culture Collection (ATCC): *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 25923), and *Proteus vulgaris* (ATCC 8427). In addition, EO samples were tested against six diagnostically confirmed MRSA clinical isolates obtained from An-Najah National University Hospital (NNUH): MRSA-1, MRSA-2, MRSA-3, MRSA-4, MRSA-5, and MRSA-6.

Fungal strains

The antifungal activity of EOs was examined against the growth of one fungal strain: *Candida albicans* (ATCC 90028).

2.3.4.2 Preparation of growth media

Bacterial growth media

Nutrient agar was prepared by dissolving 5.6 g of NA powder in 200 ml distilled water (DW). The mixture was heated to boiling point while

stirring with a magnetic stirrer on a hotplate stirrer, then autoclaved for 15 min at 121°C. The sterilization process was confirmed using sterilization indicator tape, which must turn from green to black to ensure it is sterile. After autoclaving, the sterilized solution of NA was left under aseptic conditions to cool down to around 60°C, then it was poured into suitable Petri dishes and allowed to solidify. The plates were labeled then incubated for 24h in an inverted position to prevent condensation of vapor on agar and to reduce contamination. They were then enclosed in a plastic bag, labeled, and kept in a refrigerator at 4–8°C until use. Mannitol salt agar (MSA) was prepared by dissolving 11.1 g of MSA powder in 100 ml DW, then sterilization was performed following the same procedure as for NA. Mueller Hinton broth (MHB) was prepared by dissolving 8.4 g of MHB powder in 400 ml DW. The solution was heated to the boiling point under stirring with a magnetic stirrer on a hotplate stirrer. The media mixture was autoclaved for 15 min at 121°C, and sterilization was confirmed using sterilization indicator tape. After it had cooled to a touchable temperature, the solution was poured into 50-ml sterile conical tubes then kept in a refrigerator at 4–8°C until use.

Normal saline was prepared by autoclaving 4.5g of NaCl in 500 ml DW for 15 min at 121°C to reach sterility, then it was labeled and kept in a refrigerator at 4–8°C until use.

Fungal growth media

Ready-to-use sterile RPMI 1640 medium with L-glutamine was used to culture *Candida albicans* in this study. The medium was purchased from Biological Industries.

2.3.4.3 Preparation of microorganism strains

Bacterial strains

Freshly prepared bacterial strains were used, with all strains cultured 24h before use. Initially, all tested bacterial strains, *E. coli*, *K. pneumonia*, *P. mirabilis*, *P. aeruginosa*, and *S. aureus*, were cultured on NA growth medium. Clinically confirmed MRSA and *S. aureus* isolates were cultured on MSA to confirm their identity. A concentration of 1–2 × 10^8 CFU/ml was achieved by suspending a few freshly subcultured bacterial colonies into sterile normal saline and then adjusting to the 0.5 McFarland standard. For each prepared bacterial suspension, 100 µl was mixed with 10 ml MHB, then this suspension was plated into 96-well plates.

Fungal strains

The fungal strain, *C. albicans*, was freshly cultured in sabouraud dextrose agar24h before use. A concentration of 1–5 ×10^6CFU/ml was achieved by suspending a few freshly subcultured *C. albicans* colonies into sterile NS and then adjusting to the 0.5 McFarland standard. For the

prepared *C. albicans* suspension, 100 µl was mixed with 10 ml RPMI, then this suspension was plated into 96-well plates.

2.3.4.4 Preparation of plant essential oil solutions

To obtain *C. fenzlii* EOs at an initial concentration of 400 µl/ml for bacterial and fungal assays, 40% of oil extract from each region was mixed with 60% of DMSO 100% and then mixtures were incubated for 30 min under UV light.

2.3.4.5 Antimicrobial assays

The susceptibility testing of microorganisms was performed using the broth microdilution method, as described elsewhere with some modifications [33-36].

Antibacterial assay

The broth microdilution method was used to determine the MIC, used to estimate the antibacterial activity of each EO. Using a multichannel pipette, 50 µl of sterile MHB was added to the 96-well microplate from wells 2 to 12 and from A to H. For each of the prepared plant EO solutions (400 µl/ml), 100 µl was added to well 1, then a two-fold serial dilution was conducted by transferring 50 µl from one well to the next, starting from 1 to 10 and from A to H, keeping the final volume at 50 µl. The bacterial suspension was added to the 96-well microplate from 1 to 12 and from A to G (one type of bacteria per row). Well number 11 served as the positive

control (only bacteria and media, without EO), while well 12 served as the negative control (only media, without EO). From the overnight cultures of all bacterial isolates used in this study, suspensions were prepared as mentioned above to obtain a final concentration of around 10^6CFU/ml in each well of the 96-well microplate. From well A to G, every row was utilized for one specific type of bacteria, while row H contained an oil control containing only media and serially diluted EO. The entire process was carried out under aseptic conditions. The final concentrations of *C. fenzlii* EOs ranged from 200 to 0.39 µl/ml. Plates were incubated for 20-24h at 35–37°C. Bacterial growth was verified by the presence of turbidity in the wells. The lowest concentration that did not show bacterial growth was considered the MIC.

Antifungal assay

The broth microdilution method was used to determine MIC values used to estimate the antifungal activity, using the same procedure as that used for antibacterial activity but with minor modifications. Using a multichannel pipette, 100 µl of sterile RPMI 1640 medium was added to the 96-well microplate from wells 2 to 12 and from A to H. Each pair of consecutive rows was used to test a particular type of EO. For each of the prepared plant EO solutions (400 µl/ml), 200 µl was added into well number 1, then a two-fold serial dilution was conducted by transferring 100 µl from one well into the next one, starting from 1 to 10 and from A to H, keeping the final volume at 100 µl. The fungal suspension was added to

one row but not the next to maintain a baseline for EO to which the matching well containing the fungal suspension could be compared (two rows for each EO, one containing the yeast and the other to perform serial dilutions of the EO). Well number 11 served as the positive control (only fungus and media, without EO), while well 12 served as the negative control (only media, without EO). From the overnight culture of C. *albicans* used in this study, suspensions were prepared as previously mentioned to obtain a final concentration of around 10^4CFU/ml in each well of the 96-well microplate. From wells A to H, two rows were used for each EO. The entire process was carried out under aseptic conditions. The final concentrations of *C. fenzlii* EOs ranged from 200 to 0.39 µl/ml. Plates were incubated for 48 h at 35–37°C. Fungal growth was verified by the presence of turbidity in the wells. The lowest concentrations that did not show fungal growth was considered the MIC.

2.3.5 Statistical analysis

Statistical analysis was conducted using one-way ANOVA with Turkey-Kramer HSD multiple comparison post hoc calculation, *p*-values of 0.05 or less were considered statistically significant [37].

CHAPTER THREE

CHAPTER 3

3. Results

The main goal of our study was to screen the chemical composition and the potential pharmacological activity of four *C. fenzlii* EO samples representing three regions in the West Bank of Palestine and to compare the findings.

3.1 Essential oil analysis

Essential oils of the four samples of *C. fenzlii* were extracted using hydrodistillation. The oils produced from *C. fenzlii* were colorless from some regions and yellow color from other regions, showing color variation, and all extracts had a strong and clear peppermint smell. The chemical constituents of the *C. fenzlii* plant EOs from four Palestinian regions were identified by the GC-MS method. Many molecules were found to represent almost 100% of the total EO. The chemical constituents of the Jnisinia *C. fenzlii* EO were dominated by oxygenated monoterpenoids (97.9%), including menthone (93.83%), eucalyptol (1.39%), piperitone (0.63%), ocimene (0.28%), limonene (0.85%), L-β-pinene (0.36%), beta-thujene (0.28%), alpha-pinene (0.23%) and alpha-patchoulene (0.05%). The remaining constituents were hydrocarbon sesquiterpenoids, including caryophyllene (1.64%). The chemical constituents of the Auja *C. fenzlii* EO were dominated by oxygenated monoterpenoids (99.16%), including menthone (84.54%), eucalyptol (0.31%), limonene (0.45%), l-β-pinene

(0.11%), beta-thujene (0.09%), piperitone oxide (13.31%), alpha-pinene (0.03%) and alpha-patchoulene (0.32%). The remaining constituents were hydrocarbon sesquiterpenoids, including caryophyllene (0.74%). The chemical constituents of Zawata *C. fenzlii* EO were dominated by oxygenated monoterpenoids (97.54%), including menthone (50.6%), pulegone (39.15%), eucalyptol (2.22%), piperitone (0.83%), alpha-patchoulene (0.79%), alpha-terpineol acetate (0.48%), verbenone (2.78%) and (3-beta, 5.alpha)-3,5-dihydroxy-ergost-25-ene-6,12-dione (0.69%). The remaining constituents were hydrocarbon sesquiterpenoids, including caryophyllene (0.74%). The chemical constituents of Wadiqana *C. fenzlii* EO were dominated by oxygenated monoterpenoids (99.18%), including menthone (14.83%), pulegone (79.27%), eucalyptol (2.02%), limonene (0.57%), L-β-pinene (0.14%), beta-thujene (0.65%), alpha-pinene (0.26%), alpha-patchoulene (0.36%) and verbenone (1.08%). The remaining constituents were hydrocarbon sesquiterpenoids, including caryophyllene (0.74%). The most abundant components in all of four samples were menthone and pulegone. The total percentage of identified components in the four samples was very similar, with 99.54%, 99.9%, 98.28%, and 99.92% of the constituents identified in EOs from plants collected in Jnisinia, Auja, Zawata and Wadiqana districts, respectively. Chemical analyses conducted using GC-MS characterized the EOs, with many compounds classified as oxygenated ingredients, mainly ketones, and nonoxygenated ingredients, mainly hydrocarbons, in all four samples,

although each of the compounds was present at different proportions (Table 1 and Figures 5, 6, 7, 8 and 9).

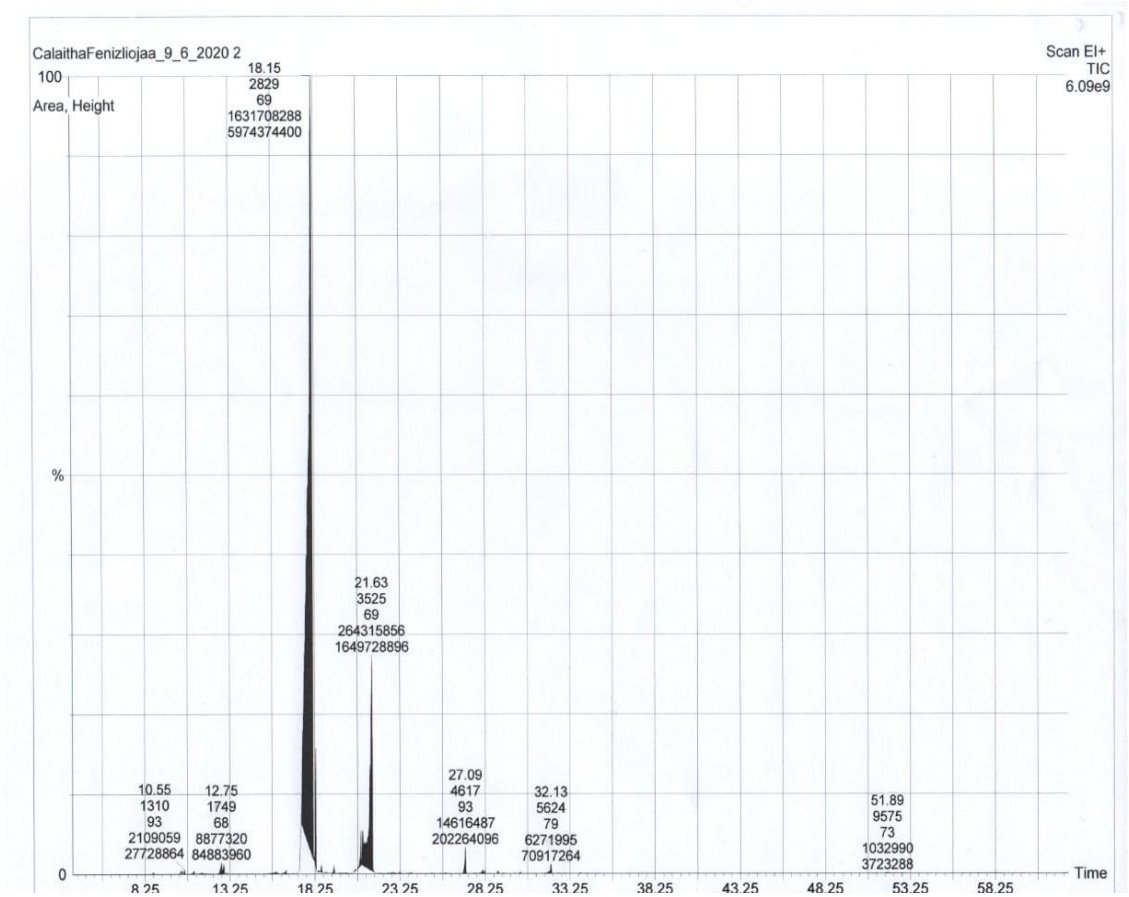

Figure 5: GC-MS result for the Auja *C. fenzlii* EO components

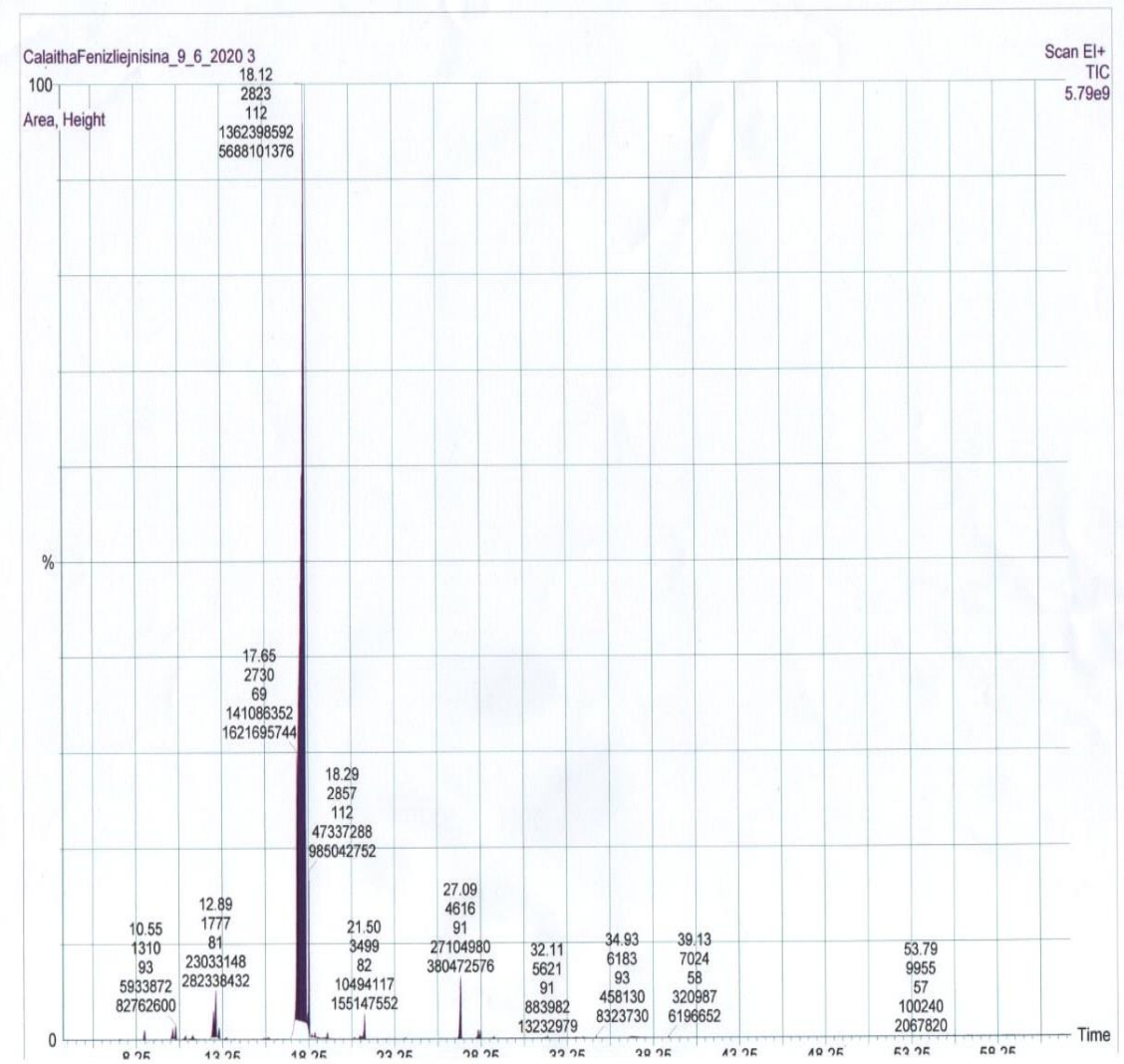

Figure 6. GC-MS result for the Jnisinia *C. fenzlii* EO components

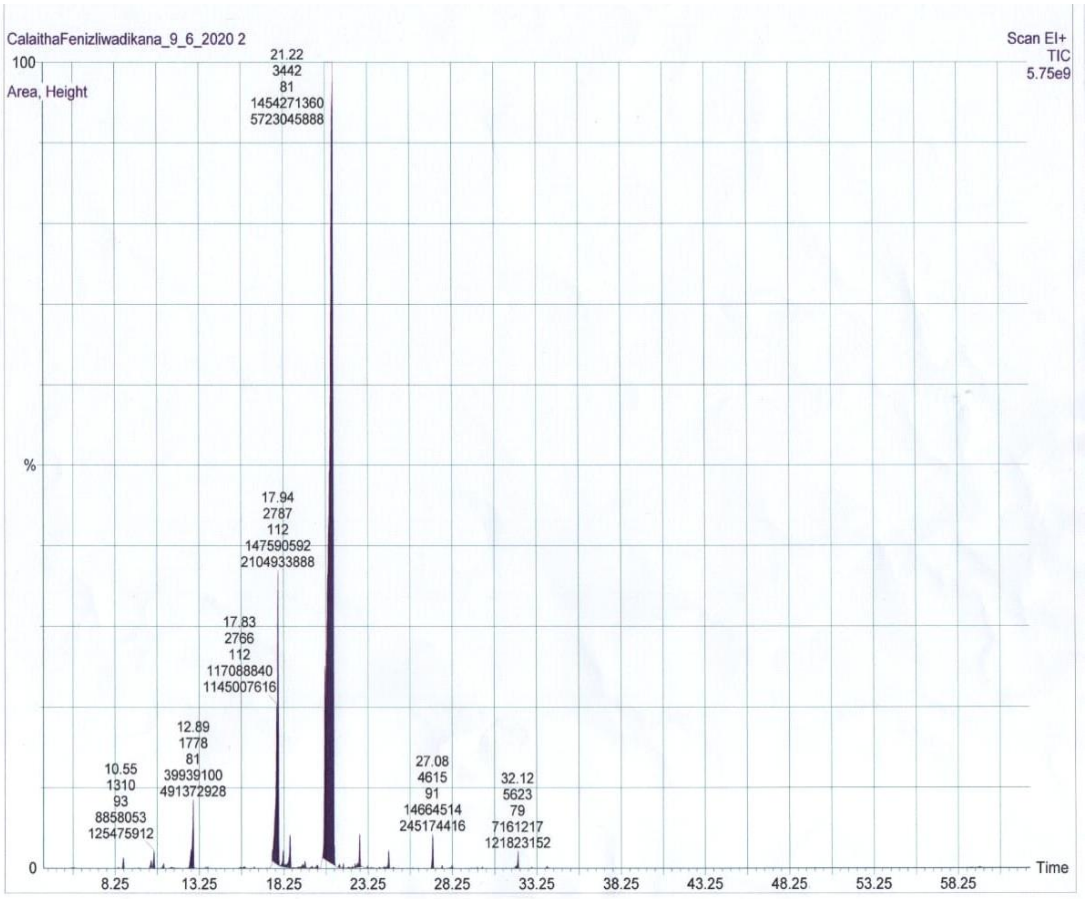

Figure 7. GC-MS result for Wadiqana *C. fenzlii* EO components

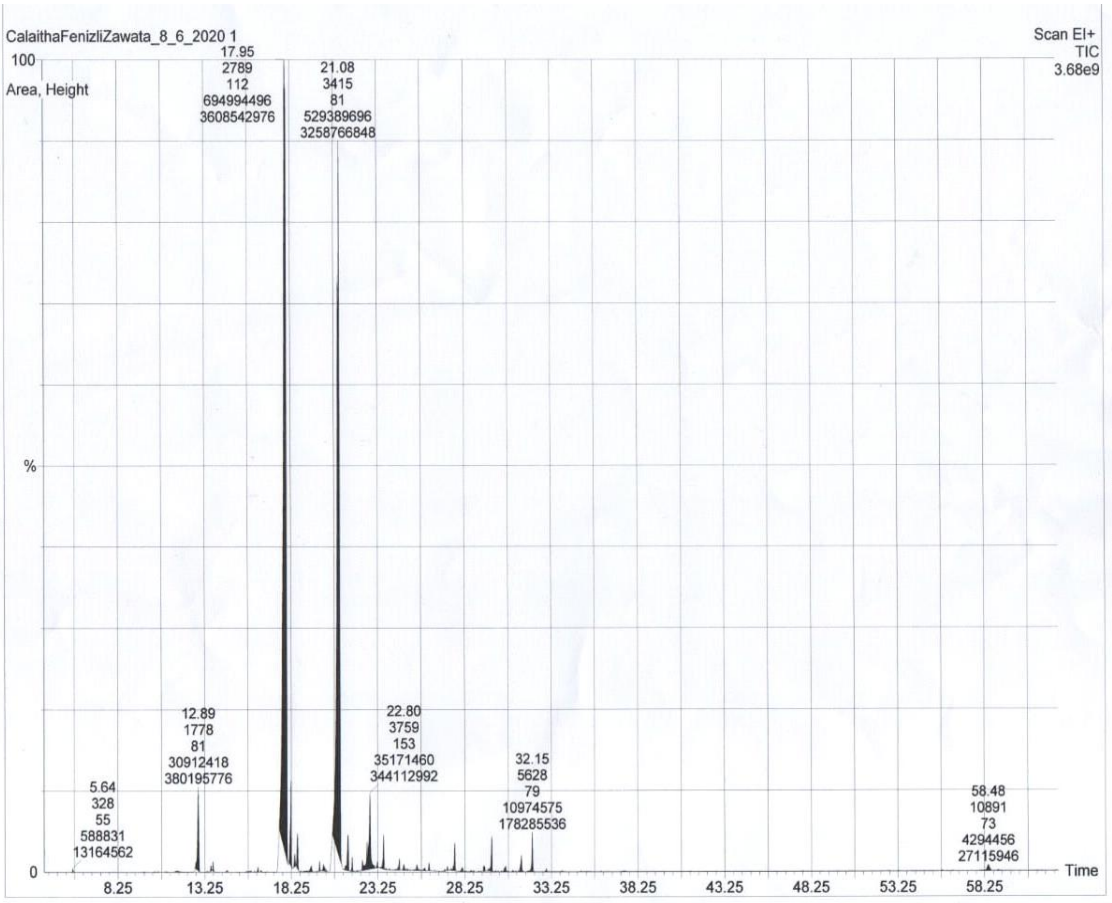

Figure 8. GC-MS result for the Zawata *C. fenzlii* EO components

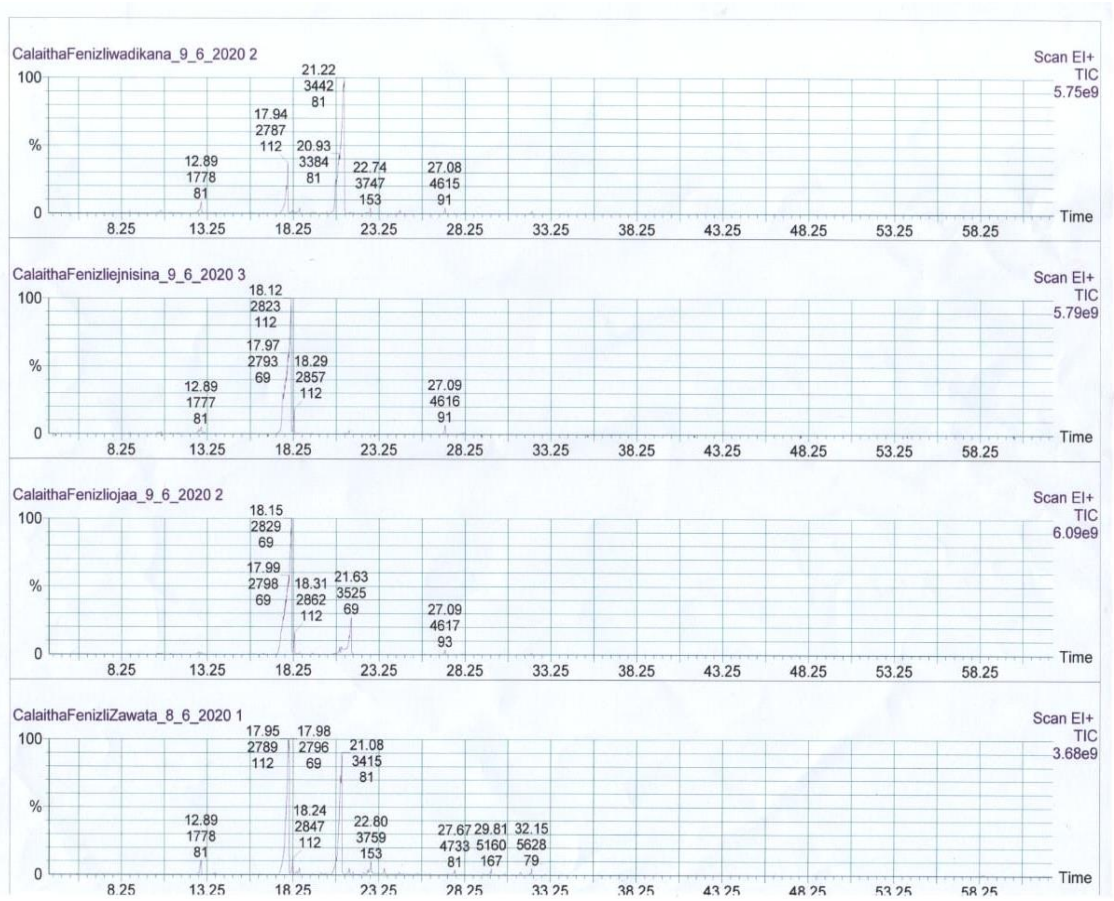

Figure 9: GC-MS result for 4 areas about their EOs components.

Table1: Chemical compounds, totally identified compounds, and chemical groups of four samples of *C. fenzlii* Eos.

Names of identified compounds	RT	RI	% total EO Jnisinia	% total EO Auja	% total EO Zawata	% total EO Wadiqana
Menthone	18	933	93.83%	84.54%	50.6%	14.83%
Pulegone	21	843	-	-	39.15%	79.27%
Eucalyptol	12.8	824	1.39%	0.31%	2.22%	2.02%
Piperitone	21.5	917	0.63%	-	0.83%	-
Ocimenes	13	890	0.35%	-	-	-
Limonene	12.75	869	0.85%	0.45%	-	.57%
L-B-Pinene	10.7	813	0.36%	0.11%	-	.14%
Beta Thujene	10.4	795	0.28%	0.09%	-	.65%
Piperitone oxide	21.6	721	-	13.31%	-	-
Alpha Pinene	8.7	831	0.23%	0.03%	-	.26
Alpha Patchoulene	32.1	867	0. 05%	0.32%	0.79%	.36%
Alpha-Terpineol acetate			-	-	0. 48%	-

Names of identified compounds	RT	RI	% total EO Jnisinia	% total EO Auja	% total EO Zawata	% total EO Wadiqana
Verbenone	22.7	789.5	-	-	2.78%	1.08%
(3-Beta,5.Alpha)-3,5-Dihydroxy-ergost-25-ene-6,12-dione	29.7	727	-	-	0.69%	-
Caryophyllene	27	896	1.64%	0.74%	0.74%	0.74%
Phytochemical classes						
Hydrocarbon sesquiterpenoid			1.64%	0.74%	0.74%	0.74%
Oxygenated Monoterpenoid			97.9%	99.16%	97.54%	99.18%
Total Identified Components %			99.54%	99.9%	98.28 %	99.92%

3.2 Antimicrobial activity

3.2.1 Antibacterial activity

The MIC values of *C. fenzlii* EOs from different regions of Palestine are reported in Table 2. All five bacterial ATCC strains, in addition to the six diagnostically confirmed MRSA strains used in this study, were sensitive to *C. fenzlii* EOs, with MICs ranging from 50 to 12.5 µl/ml. There were differences in activity against 11 microbial strains between *C. fenzlii* EOs from the four regions in Palestine. The antibacterial activity of each EO sample against the ATCC strains showed no differences between strains, and the EO sample from *Calamintha fenzlii* Wadiqana (CFW) had the highest potency, with a MIC value of 12.5 µl/ml. The CFJ EO sample had the lowest potency (MIC of 50 µl/ml) compared to the other EO samples against the six clinical MRSA isolates, with a potency that ranged

between 12.5 and 50µl/ml. In general, the potency of EO samples against clinical strains showed lower MIC potency compared to the ATCC strains.

Table 2. Antimicrobial activity (minimum inhibitory concentration [MIC] in µl/ml) of Calamintha fenzlii EOs from different regions of Palestine based on the broth microdilution method.

	MIC CFJ	**MIC CFA**	**MIC CFZ**	**MIC CFW**
Yeast				
***C. albicans* (ATCC 90028)**	25	R	12.5	25
Bacterial strains				
***S. aureus* (ATCC 25923)**	25	25	25	12.5
***E. coli* (ATCC 25922)**	25	25	25	12.5
***P. aeruginosa* (ATCC 9027)**	25	25	25	12.5
***K. pneumonia* (ATCC 13883)**	25	25	25	12.5
***P. vulgaris* (ATCC 8427)**	25	25	25	12.5
MRSA-1	25	12.5	25	12.5
MRSA-2	25	25	25	25
MRSA-3	50	25	25	25
MRSA-4	50	25	25	25
MRSA-5	50	25	50	25
MRSA-6	50	25	50	25

3.2.2 Antifungal activity

The fungal strain was tested for sensitivity to *C. fenzlii* EOs. *C. albicans* (ATCC 90028) was found to be sensitive to *C. fenzlii* EO samples, MIC values ranged from 12.5 to 25 µl/ml, except for CFA in which *C. albicans* was found resistant (Table 2).

CHAPTER FOUR

CHAPTER 4

4. Discussion

4.1 Chemical analysis

The GC-MS analysis, performed under the conditions mentioned above, identified many compounds, listed in Table 1. The results of the chromatographic profiles of the four *C. fenzlii* EO samples were dominated by oxygenated ingredients including ketones, with a range of 97.5–99.18%, and hydrocarbons, ranging from 0.74–1.6%. Among the oxygenated compounds, menthone was the predominant compound, ranging from 14.8–93.8%, and pulegone was the second most abundant component, ranging from 0–79.2%. Less than 2% of nonoxygenated components were detected, present in the form of caryophyllene. Studies previously conducted on *C. fenzlii* EOs from Palestine reported 68.9% menthone and 23.1% pulegone (Table 3) [4]. For plants grown in Turkey, isomenthone (42.1%), pulegone (28.9%), and piperitenone (11.8%) were detected in EO obtained by steam distillation (Table 3) [38]. For *C. fenzlii* grown in Morocco, pulegone was found to be the main component, with a percentage of 78.07% (Table 3) [39]. In Tunis, pulegone was the main component, with a percentage of 44.27%, followed by isomenthone (19.05%) (Table3) [40]. For plants grown in Iran, the prominent components were piperitone (38.0%) and piperitenone (33.0%) (Table3) [41]. Finally, for plants grown in Bulgaria, pulegone was the main component, with a percentage ranging from 42.9–

45.4%, followed by piperitenone (21.7–23.1%) and isomenthone (11.3–12.8%) (Table3) [42].

The remaining components detected in the current study, including eucalyptol, piperitone, ocimene, limonene, l-β-pinene, beta-thujene, alpha-pinene, alpha-patchoulene, alpha-terpineol acetate, verbenone and caryophyllene, were present at higher levels than those previously detected in Palestine, Turkey, Tunis, and Morocco, and many were not even identified in previous samples. The differences in chemical compounds may be explained by variations in environmental conditions, including location, climate, seasonal and geographical factors [25]. It is also dependent on the part of the plant studied and the growth period of leaves, as younger leaves were previously found to be rich in pulegone, which accounted for 70% of the EO [25].

Table 3. Overview of the main components of *Calamintha fenzlii* EOs from different origins, as reported by previous studies.

	Palestine	Turkey	Morocco	Tunis	Iran	Bulgaria
Menthone	68.9%	-	-	-	-	-
Pulegone	23.1%	28.9%	78.07%	44.27%	2.3%	42.9-45.4%
Isomenthone	-	42.1 %	-	19.05%	-	11.3-12.8%
Piperitenone	-	11.8 %	-	-	33.0%	21.7-23.1%
Piperitone	-	-	-	-	38.0%	-

It has been found *Mentha pulegium* L. oil from Bulgaria contains pulegone (42.9-45.4%); from Uruguay; pulegone (73.4%), isomenthone

(12.9%); from Egypt; pulegone (43.5%), piperitone (12.2%); from Tunisia, pulegone (41.8%), isomenthone (11.3%). These studies showed three chemotypes of Menthapulegium L. with the following major oil components (1) pulegone, (2) piperitenone and/or piperitone, and (3) isomenthone/neoisomenthol [43].

4.2 Antimicrobial activity:

Multidrug-resistant bacterial species cause health difficulties. Extracts of EOs have been investigated as new potential antimicrobial agents, bio preservative products, and promising antiseptic enhancer for topical use [25]. Based on our results, *C. fenzlii* EOs can be considered strong broad-spectrum antimicrobial agents. The antimicrobial properties of four *C. fenzlii* EO samples from different regions of Palestine were examined against 11 bacterial strains (five ATCC strains and six clinically confirmed MRSA strains), in addition to one yeast ATCC strain. The antimicrobial activity was based on MIC values determined using the microdilution method. The results listed in Table 2 show that the EOs of the four samples exhibited considerable antifungal and antibacterial potency. The results for antimicrobial activity of the four EO samples revealed that this activity was specific and strong against fungi and bacteria, but the antimicrobial potency was slightly lower in cases of clinically confirmed MRSA strains and some fungi. In a study performed in Iran, the antimicrobial activity of *C. fenzlii* EO was investigated according to the type of organism, and significant antibacterial activity was

discovered against Gram-positive bacteria, especially *S. aureus*, but there was more resistance to Gram-negative bacteria, especially *E.coli* [41]. In Iran, people have traditionally used the *Mentha pulegium* L. plant against infectious illness, and it was reported to efficacious against these problems without any scientific basis to explain this action. This study shows evidence of the antimicrobial activity of this oil. The high concentration of piperitone and its synergistic effect with other components may explain the traditional use of *M. pulegium* L. for treating microbe-related disease. Therefore, it shows potential for use as an alternative to antibiotics, especially as it has no proven side effects [41]. The antibacterial activity of EOs is related to their chemical composition. The oil was found to be rich in oxygenated monoterpenes, which show higher antibacterial activity than oils rich in monoterpene hydrocarbons. The antibacterial activity of *C. fenzlii* EO from Algeria can be attributed to the high percentage of pulegone and piperitone; therefore, these oxygenated monoterpene compounds are responsible for the antibacterial activity [44]. These findings are similar to our results for plants obtained from Wadiqana, as the EO of *C. fenzlii* from this area showed the highest quantity of pulegone (79.27%), which is thought to be an effective antibacterial agent [45].

CHAPTER FIVE

CHAPTER 5

5. Conclusion

Calamintha fenzlii EOs from four different regions in Palestine showed variable antimicrobial activities depending on the phytochemical composition of the EO. The EOs from the four regions studied had the same major chemical components but in different proportions. The plant extracts exhibited strong antifungal and antibacterial activities. The sample from Wadiqana showed the highest potency against ATCC strains, while the sample from Auja showed lower potency against MRSA. These findings suggest that *C. fenzlii* EO is a promising agent for treating diseases caused by bacteria and fungi, especially those that have developed antimicrobial resistance.

5.1 Recommendations

Future work may include:

- Further *in vivo* studies are needed to evaluate the potential antimicrobial activity of EOs.
- Further studies are required to isolate the basic components responsible for the potential antibacterial activity.
- Further studies are required to evaluate the safety and toxicity of plant EOs.

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جامعة النجاح الوطنية

كلية الدراسات العليـــا

التأثير الدوائي واختلاف المكونات الكيميائية للزيت المستخلص من النعنع البري في ثلاث مناطق مختلفة في فلسطين

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في علم الصيدلة السريرية من كلية الدراسات العليا في جامعة النجاح الوطنية في نابلس- فلسطين.

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الملخص

الخلفية والأهداف:

زيت النعنع البري هو واحد من النباتات العطرية الطبية التي تهيمن على مناطق شرق البحر الأبيض المتوسط بما في ذلك فلسطين، ويمتلك رائحة نعنع عطرية لطيفة . الهدف من الدراسة الحالية هو فحص ومقارنة المكونات الكيميائية والخصائص المحتملة لزيت النعنع البري الذي تم جمعه من ثلاث مناطق مختلفة في الضفة الغربية – فلسطين.

الأساليب :

تم استخراج الزيوت الرئيسية من اربع عينات نعنع بري باستخدام جهاز التقطير المائي hydrodistillation. تم تحليل العينات للكشف عن مكوناتها الكيميائية بواسطة استخدام GC–MS تم فحص الفاعلية المضادة للميكروبات باستخدام طريقة broth microdilution ، واستخدمت 11 سلالة بكتيرية؛ سبعة منها إيجابية غرام *Staphylococcus aureus* : وستة من أصل هذه السبعة هي عينات من *Staphylococcus aureus* methicillin–resistant [MRSA]أما السلالات الاربعة الباقية فهي سالبة غرام و تنتمي الى الأنواع التالية:

Proteus vulgaris, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae,

كما تمت الاستعانة بنوع واحد من الفطريات وهو*Candida albicans*

النتائج:

أظهر تحليل GC-MS النسب المئوية العالية للمكونات المؤكسجة التي تراوحت بين (97.54-99.18)٪، أما المكونات غير المؤكسجة، فكانت تتراوح بين1.64-0.74 ٪، (وكان مجموع المركبات التي تمكنت الدراسة من كشفها و دراستها ما بين99.9-98.28) ٪ (ولوحظت عدة مكونات، وكان menthone المكون الأكثر وفرة في العينات الأربعة حيث تراوح بين 93.83-14.83)٪ (وأيضا pulegone الذي كان أكثر المكونات وفرة في اثنتين من العينات حيث تراوح ما بين 79.27-39.15) ٪).وقد أظهرت العينات الأربع نشاطاً واسع النطاق لمضادات الميكروبات؛ وأظهرت ثلاث عينات نشاطا مضادا للفطريات حيث ان تركيز الحد الادنى للتثبيط تراوح ما بين 25-12.5) ميكرولتر /مل (بينما لم تظهر العينة الباقية اي نشاط مضاد للفطريات. وأظهرت عينة من وادي قانا أعلى نشاط ضد العينات البكتيرية المرجعية الخمس المستخدمة (ATCC) مع اقل تركيز اظهر الحد الادنى للتثبيط12.5) ميكرولتر/مل (وأظهرت عينة اخرى من جنيسنيا اقل نشاط ضد بكتيريا (MRSA) مع اعلى تركيز للحد الادنى للتثبيط 50)ميكرولتر/مل) على كل حال، اظهرت العينات الاربع نشاطا واسع النطاق ضد الميكروبات مع تركيز الحد الادنى للتثبيط ما بين (50-12.5) ميكرولتر/مل.

الخلاصة :

أظهرت الدراسة أن عينات زيت النعنع البري من مناطق مختلفة في فلسطين تحتوي على نسب مختلفة من المواد الكيميائية التي تمتلك أنشطة بيولوجية محتملة مختلفة مثل الأنشطة المضادة للميكروبات والتي تتماشى مع الاستخدامات التقليدية للمستخلصات النباتية. وأظهرت المستخلصات النباتية فعالية مضادة للبكتيريا أعلى من تلك التي تمتلكها النباتات المرجعية، ويلزم إجراء مزيد من الدراسات لتقييم الأنشطة الدوائية المحتملة، والسلامة والسمية للمستخلصات النباتية و تفاعلاتها داخل الجسم . كما أن هناك حاجة إلى مزيد من الدراسات لعزل وتحديد وتوصيف المكونات الرئيسية المسؤولة عن الأنشطة الدوائية المحتملة لنبتة النعنع البري.